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(54) **PEPTIDES EFFECTIVE IN THE TREATMENT OF CONDITIONS REQUIRING THE REMOVAL OR DESTRUCTION OF CELLS**

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None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

6,660,830	B1 *	12/2003	Radulescu	C07K 14/4703 530/300
7,642,063	B2 *	1/2010	Samoylova	G01N 33/5011 424/1.49
8,303,957	B2 *	11/2012	Firat	C07K 14/4748 424/184.1

* cited by examiner

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(57) **ABSTRACT**

The embodiments are directed to peptides, compositions, and methods of treating conditions requiring removal or destruction of harmful or unwanted cells in a patient, such as benign and malignant tumors, using compounds containing or based on peptides.

13 Claims, No Drawings

PEPTIDES EFFECTIVE IN THE TREATMENT OF CONDITIONS REQUIRING THE REMOVAL OR DESTRUCTION OF CELLS

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Jul. 16, 2014, is named 063307-0428298_SL.txt and is 998 bytes in size.

FIELD

The present invention is directed to methods of treating conditions requiring removal or destruction of cellular elements, such as benign or malignant tumors in humans, using compounds based on peptides. The method includes, but is not limited to, administering the compounds, or salt forms thereof, intramuscularly, orally, intravenously, intrathecally, intratumorally, intraprostatically, intranasally, topically, transdermally, etc., either alone or conjugated to a carrier.

DESCRIPTION OF RELATED ART

The essence of many medical treatments and procedures involves the removal or destruction of harmful or unwanted tissue. Examples of such important treatments include the surgical removal of cancerous growths, the destruction of metatastic tumors through chemotherapy, and the reduction of glandular (e.g. prostate) hyperplasia. Other examples include the removal of unwanted facial hair, the removal of warts, and the removal of unwanted fatty tissue.

There is a need for an effective agent that can destroy and hence either facilitate the removal of or inhibit the further growth of harmful or unwanted cells and tissue. There also is a need for such an agent that primarily has only local effects and minimal or no systemic toxicity.

Certain peptides have been shown to have potential utility in treating tumors and other conditions requiring removal or destruction of cells, such as the peptides as disclosed in the following U.S. Pat. No. 7,192,929; U.S. Pat. No. 7,241,738; U.S. Pat. No. 7,317,077; and U.S. Pat. No. 7,408,021. Disclosed herein are certain peptides that also are useful in treating tumors and other conditions requiring removal or destruction of cells.

Cancer is an abnormality in a cell's internal regulatory mechanisms that results in uncontrolled growth and reproduction of the cell. Normal cells make up tissues, and when these cells lose their ability to behave as a specified, controlled, and coordinated unit, (dedifferentiation), the defect leads to disarray amongst the cell population. When this occurs, a tumor is formed.

Benign overgrowths of tissue are abnormalities in which it is desirable to remove cells from an organism. Benign tumors are cellular proliferations that do not metastasize throughout the body but do, however, cause disease symptoms. Such tumors can be lethal if they are located in inaccessible areas in organs such as the brain. There are benign tumors of organs including lung, brain, skin, pituitary, thyroid, adrenal cortex and medulla, ovary, uterus, testis, connective tissue, muscle, intestines, ear, nose, throat, tonsils, mouth, liver, gall bladder, pancreas, prostate, heart, and other organs.

Surgery often is the first step in the treatment of cancer. The objective of surgery varies. Sometimes it is used to remove as much of the evident tumor as possible, or at least to "debulk" it (remove the major bulk(s) of tumor so that there is less that needs to be treated by other means). Depending on the cancer type and location, surgery may also provide some symptomatic relief to the patient. For instance, if a surgeon can remove a large portion of an expanding brain tumor, the pressure inside the skull will decrease, leading to improvement in the patient's symptoms.

Not all tumors are amenable to surgery. Some may be located in parts of the body that make them impossible to completely remove. Examples of these would be tumors in the brainstem (a part of the brain that controls breathing) or a tumor which has grown in and around a major blood vessel. In these cases, the role of surgery is limited due to the high risk associated with tumor removal.

For some tumors, surgery is available as an active treatment option but may cause complications and significant short-term and long-term adverse events that may outweigh the benefits of the surgery to the patient. For example, radical prostatectomy is a commonly used treatment option for localized low-grade prostate cancer detected through prostate-specific antigen (PSA) screening and biopsy but can cause short- and long-term adverse effects, including impotence, urinary dysfunction, and other complications, particularly when weighed against the low-risk such cancers can represent to the patient.

In some cases, surgery is not used to debulk tumor because it is simply not necessary. An example is Hodgkin's lymphoma, a cancer of the lymph nodes that responds very well to combinations of chemotherapy and radiation therapy. In Hodgkin's lymphoma, surgery is rarely needed to achieve cure, but almost always used to establish a diagnosis.

Chemotherapy is another common form of cancer treatment. Essentially, it involves the use of medications (usually given by mouth or injection) which specifically attack rapidly dividing cells (such as those found in a tumor) throughout the body. This makes chemotherapy useful in treating cancers that have already metastasized, as well as tumors that have a high chance of spreading through the blood and lymphatic systems but are not evident beyond the primary tumor. Chemotherapy may also be used to enhance the response of localized tumors to surgery and radiation therapy. This is the case, for example, for some cancers of the head and neck.

Unfortunately, other cells in the human body that also normally divide rapidly (such as the lining of the stomach and hair) also are affected by chemotherapy. For this reason, many chemotherapy agents induce undesirable side effects such as nausea, vomiting, anemia, hair loss or other symptoms. These side effects are temporary, and there exist medications that can help alleviate many of these side effects. As our knowledge has continued to grow, researchers have devised newer chemotherapeutic agents that are not only better at killing cancer cells, but that also have fewer side effects for the patient.

Chemotherapy is administered to patients in a variety of ways. Some include pills and others are administered by an intravenous or other form of injection. For injectable chemotherapy, a patient goes to the doctor's office or hospital for treatment. Other chemotherapeutic agents require continuous infusion into the bloodstream, 24 hours a day. For these types of chemotherapy, a minor surgical procedure is performed to implant a small pump worn by the patient. The pump then slowly administers the medication. In many cases, a permanent port is placed in a patient's vein to eliminate the requirement of repeated needle sticks.

Radiation therapy is another commonly used weapon in the fight against cancer. Radiation kills cancer by damaging the DNA within the tumor cells. The radiation is delivered in different ways. The most common involves pointing a beam

of radiation at the patient in a highly precise manner, focusing on the tumor. To do this, a patient lies on a table and the beam moves around him/her. The procedure lasts minutes, but may be done daily for several weeks (depending on the type of tumor), to achieve a particular total prescribed dose.

Another radiation method sometimes employed, called brachytherapy, involves taking radioactive pellets (seeds) or wires and implanting them in the body in the area of the tumor. The implants can be temporary or permanent. For permanent implants, the radiation in the seeds decays over a period of days or weeks so that the patient is not radioactive. For temporary implants, the entire dose of radiation is usually delivered in a few days, and the patient must remain in the hospital during that time. For both types of brachytherapy, radiation is generally delivered to a very targeted area to gain local control over a cancer (as opposed to treating the whole body, as chemotherapy does.)

Some highly selected patients may be referred for bone marrow transplants. This procedure usually is performed either because a patient has a cancer that is particularly aggressive or because they have a cancer that has relapsed after being treated with conventional therapy. Bone marrow transplantation is a complicated procedure. There are many types, and they vary in their potential for causing side effects and cure. Most transplants are performed at special centers, and in many cases, their use is considered investigational.

A number of other therapies exist, although most of them are still being explored in clinical trials and have not yet become standard care. Examples include the use of immunotherapy, monoclonal antibodies, anti-angiogenesis factors and gene therapy.

Benign tumors and malformations also can be treated by a variety of methods including surgery, radiotherapy, drug therapy, thermal or electric ablation, cryotherapy, and others. Although benign tumors do not metastasize, they can grow large and they can recur. Surgical extirpation of benign tumors has all the difficulties and side effects of surgery in general and oftentimes must be repeatedly performed for some benign tumors, such as for pituitary adenomas, meningeomas of the brain, prostatic hyperplasia, and others.

Other conditions involving unwanted cellular elements exist where selective cellular removal is desirable. For example, heart disease and strokes commonly are caused by atherosclerosis, which is a proliferative lesion of fibrofatty and modified smooth muscle elements that distort the blood vessel wall, narrow the lumen, constrict blood flow, predispose to focal blood clots, and ultimately lead to blockage and infarction. There are various treatments for atherosclerosis such as bypass grafts; artificial grafts; angioplasty with recanalization, curettage, radiation, laser, or other removal; pharmacotherapy to inhibit atherosclerosis through lipid reduction; anti-clotting therapies; and general measures of diet, exercise, and lifestyle. A method for removing atherosclerotic lesions without the risk and side effects of surgical procedures is needed.

Other examples of unwanted cellular elements where selective cellular removal is desirable include viral induced growths, such as warts. Another example is hypertrophic inflammatory masses found in inflammatory conditions, such as tonsillitis and adenoiditis, and hypertrophic scars or keloids. Still other examples are found in cosmetic contexts such as the removal of unwanted hair, e.g., facial hair, or for shrinkage of unwanted tissue areas for cosmetic purposes, such as in the facial dermis and connective tissues or in the dermas and connective tissue of the extremities.

Other examples of unwanted cellular elements where selective cellular removal or the inhibition of cellular proliferation is desirable include stenosis and restenosis of any artery, valve or canal in the circulatory system including, but not limited to, valves (e.g., aortic stenosis which involves narrowing of the aortic valve orifice), coronary arteries (e.g., coronary ostial sclerosis which involves narrowing of the mouths of the coronary arteries), carotid arteries, and renal arteries. Other examples include the inhibition or removal of unwanted cellular growth or accumulation causing the partial or complete occlusion of medical devices such as stents placed or implanted within a blood vessel for treating stenoses, strictures or aneurysms therein or within the urinary tract and in bile ducts.

Still other examples will be obvious to those of ordinary skill in the art. In all or most of these examples there is a need for treatments that can remove or destroy the unwanted cellular elements without the risks and side effects of conventional therapies or remove the unwanted cellular elements with more precision.

There remains a need in the art for new, less toxic treatments for treating unwanted cellular elements. The present invention satisfies these needs.

SUMMARY

The embodiments described herein are premised in part on the discovery that certain newly discovered peptides are capable of treating and/or killing unwanted cellular proliferations, such as benign and malignant tumors, glandular (e.g. prostate) hyperplasia, hypertrophic inflammatory masses, unwanted facial hair, warts, and unwanted fatty tissue. Embodiments also relate to methods of treating unwanted cellular proliferations comprising administering to a mammal in need thereof a therapeutically effective amount of one or more the peptides disclosed herein.

Such a peptide compositions can be administered alone or conjugated to a carrier or an antibody. The peptide can be administered intramuscularly, orally, intravenously, intraperitoneally, intracerebrally (intraparenchymally), intracerebroventricularly, intratumorally, intraprostatically, intralesionally, intradermally, intrathecally, intranasally, intraocularly, intraarterially, topically, transdermally, via an aerosol, infusion, bolus injection, implantation device, sustained release system etc., either alone or conjugated to a carrier. Alternatively, the peptide can be expressed in vivo by administering a gene that expresses the peptide or by introducing cells, bacteria or viruses that express the peptide in vivo, because of genetic modification or otherwise. In addition, the peptide may be used in conjunction with other therapies for treating benign and malignant tumors and other unwanted or harmful cellular growths.

Both the foregoing general description and the following detailed description are exemplary and explanatory and are intended to provide further explanation of the invention as claimed. Other objects, advantages, and features will be readily apparent to those skilled in the art from the following detailed description of the invention.

DETAILED DESCRIPTION

Before the present peptides and methods are described, it is understood that the embodiments described herein are not limited to the particular methodology, protocols, cell lines, vectors, and reagents described, as these may vary. It also is to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

Terms and phrases used herein are defined as set forth below unless otherwise specified. Throughout this description, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a host cell" includes a single host cell as well as a plurality of such host cells, and a reference to "an antibody" is a reference to one or more antibodies and equivalents thereof known to those skilled in the art, and so forth.

Amino acids and amino acid residues described herein may be referred to according to the accepted one or three-letter code provided in the table below. Unless otherwise specified, these amino acids or residues are of the naturally occurring L stereoisomer form.

TABLE 1

Amino Acid	One-Letter Symbol	Three-Letter Symbol
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamine	Q	Gln
Glutamic acid	E	Glu
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

The embodiments relate to a composition comprising one or more of the peptides as defined below in this invention.

The peptides useful in the embodiments (hereinafter "Peptide") may be selected from:

(i) Ile-Asp-Leu-Leu-Gln-Gly-Arg-Thr-Arg-Asn-Arg-Cys (IDLLQGRTRNRC) (SEQ ID NO. 1), referred to in the examples below as NYMAP1385; and

(ii) Phe-His-Asp-Leu-Lys-Lys-His-Cys-Ile-Lys (FHDLKKHCIK) (SEQ ID NO. 2), referred to in the examples below as NYMAP13134.

The term "Peptide" as used herein, also encompasses as defined further below dimers, trimers and other multimers of Peptides, fragments, variants, derivatives, homologues, fusion proteins and mimetics of the Peptides, and the Peptides with (or in the form of) a pharmaceutically acceptable salt or salts.

It will be apparent to one of skill in the art that other smaller fragments of the Peptides may be selected such that these smaller fragments will possess the same or similar biological activity. Other fragments of the Peptides may be selected by one skilled in the art such that these smaller fragments will possess the same or similar biological activity. The Peptides of the embodiments encompass these other fragments.

The expression "pharmaceutically acceptable salts" of a Peptide or of a compound of the embodiments include, but are not limited to, acid addition salts formed by reacting the Peptides or compounds with pharmaceutically acceptable acids such as an inorganic acid (e.g., hydrochloric acid, phosphoric acid, hydrobromic acid, sulfuric acid) or an organic acid (e.g., acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid, benzenesulfonic acid, embonic (pamoic) acid) and include inorganic acid salts such as hydrochlorides, hydrobromides, phosphates and sulfates and organic acid salts such as trifluoroacetates, acetates, formates, citrates, tartrates, maleates, fumarates, succinates, embonates (pomates), methanesulfonates, fumarates, gluconates, tannates, benzoates, alginates, ascorbates, and glucuronates.

The Peptides described herein also include homologues, fragments, derivatives, variants, fusion proteins, and peptide mimetics of the Peptides, unless the context indicates otherwise.

The term "fragment" refers to a protein or polypeptide that consists of a continuous subsequence of the amino acid sequence of a Peptide and includes naturally occurring fragments such as splice variants and fragments resulting from naturally occurring in vivo protease activity. Such a fragment may be truncated at the amino terminus, the carboxy terminus, and/or internally (such as by natural splicing). Such fragments may be prepared with or without an amino terminal methionine. The term "fragment" includes fragments, whether identical or different, from the Peptide with a contiguous amino acid sequence in common or not, joined together, either directly or through a linker.

The term "variant" refers to a protein or polypeptide in which one or more amino acid substitutions, deletions, and/or insertions are present as compared to the amino acid sequence of the Peptides described herein and includes naturally occurring allelic variants or alternative splice variants of a Peptide. The term "variant" includes the replacement of one or more amino acids in a peptide sequence with a similar or homologous amino acid(s) or a dissimilar amino acid(s). There are many scales on which amino acids can be ranked as similar or homologous. (Gunnar von Heijne, *Sequence Analysis in Molecular Biology*, p. 123-39 (Academic Press, New York, N.Y. 1987.) Preferred variants include alanine substitutions at one or more of amino acid positions. Other preferred substitutions include conservative substitutions that have little or no effect on the overall net charge, polarity, or hydrophobicity of the protein. Conservative substitutions are set forth in Table 2 below.

TABLE 2

Conservative Amino Acid Substitutions	
Basic:	Arginine
	Lysine
	Histidine
Acidic:	Glutamic acid
	Aspartic acid
Uncharged Polar:	Glutamine
	Asparagine
	Serine
	Threonine
	Tyrosine
Non-Polar:	Phenylalanine
	Tryptophan
	Cysteine
	Glycine
	Alanine
	Valine
	Proline
	Methionine
	Leucine
	Isoleucine

Table 3 sets out another scheme of amino acid substitution:

TABLE 3

Original Residue	Substitutions
Ala	Gly; Ser
Arg	Lys
Asn	Gln; His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Ala; Pro
His	Asn; Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Tyr; Ile
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

Other variants can consist of less conservative amino acid substitutions, such as selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. The substitutions that in general are expected to have a more significant effect on function are those in which (a) glycine and/or proline is substituted by another amino acid or is deleted or inserted; (b) a hydrophilic residue, e.g., seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g., leucyl, isoleucyl, phenylalanyl, valyl, or alanyl; (c) a cysteine residue is substituted for (or by) any other residue; (d) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) a residue having an electronegative charge, e.g., glutamyl or aspartyl; or (e) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having such a side chain, e.g., glycine. Other variants include those designed to either generate a novel glycosylation and/or phosphorylation site(s), or those designed to delete an existing glycosylation and/or phosphorylation site(s). Variants include at least one amino acid substitution at a glycosylation site, a proteolytic cleavage site and/or a cysteine residue. Variants also include Peptides with additional amino acid residues before or after the peptide amino acid sequence on linker peptides. For example, a cysteine residue may be added at both the amino and carboxy terminals of a Peptide in order to allow the cyclisation of the peptide by the formation of a di-sulphide bond. The term "variant" also encompasses polypeptides that have the amino acid sequence of a Peptide with at least one and up to 25 or more additional amino acids flanking either the 3' or 5' end of the peptide.

The term "derivative" refers to a chemically modified protein or polypeptide that has been chemically modified either by natural processes, such as processing and other post-translational modifications, but also by chemical modification techniques, as for example, by addition of one or more polyethylene glycol molecules, sugars, phosphates, and/or other such molecules to the Peptide. Derivatives include salts. Such chemical modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature, and they are well known to those of skill in the art. It will be appreciated that the same type of modification may be present in the same or varying degree at several sites in a given protein or polypeptide. Also, a given protein or polypeptide may contain many types of modifications. Modifications can occur anywhere in a protein or polypeptide, including the peptide backbone, the amino acid side-chains, and the amino or carboxyl termini. Modifications include, for example, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins, such as arginylation, and ubiquitination. See, for instance, *Proteins—Structure And Molecular Properties,* 2nd Ed., T. E. Creighton, W.H. Freeman and Company, New York (1993) and Wold, F., "Posttranslational Protein Modifications Perspectives and Prospects," pgs. 1-12 in *Posttranslational Covalent Modification Of Proteins*, B. C. Johnson, Ed., Academic Press, New York (1983); Seifter et al., Meth. Enzymol. 182:626-646 (1990) and Rattan et al., "Protein Synthesis: Posttranslational Modifications and Aging," *Ann. N. Y. Acad. Sci.* 663: 48-62 (1992). The term "derivatives" include chemical modifications resulting in the protein or polypeptide becoming branched or cyclic, with or without branching. Cyclic, branched and branched circular proteins or polypeptides may result from post-translational natural processes and may be made by entirely synthetic methods, as well.

The term "homologue" refers to a protein that is at least 60 percent identical in its amino acid sequence of a Claimed Peptide, as the case may be, as determined by standard methods that are commonly used to compare the similarity in position of the amino acids of two polypeptides. The degree of similarity or identity between two proteins can be readily calculated by known methods, including but not limited to those described in *Computational Molecular Biology*, Lesk, A. M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D. W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data*, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo H. and Lipman, D., SIAM, *J. Applied Math.,* 48: 1073 (1988). Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs.

Preferred computer program methods useful in determining the identity and similarity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., *Nucleic Acids Research,* 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA, Atschul, S. F. et al., *J. Molec. Biol.,* 215: 403-410 (1990). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894; Altschul, S., et al., *J. Mol. Biol.,* 215: 403-410 (1990). By way of example, using a computer algo-

rithm such as GAP (Genetic Computer Group, University of Wisconsin, Madison, Wis.), the two proteins or polypeptides for which the percent sequence identity is to be determined are aligned for optimal matching of their respective amino acids (the "matched span", as determined by the algorithm).

A gap opening penalty and a gap extension penalty as well as a comparison matrix such as PAM 250 or BLOSUM-62 are used in conjunction with the algorithm. A standard comparison matrix (see Dayhoff et al. in: *Atlas of Protein Sequence and Structure*, vol. 5, supp. 3 [1978] for the PAM250 comparison matrix; see Henikoff et al., *Proc. Natl. Acad. Sci. USA,* 89:10915-10919 [1992] for the BLOSUM 62 comparison matrix) also may be used by the algorithm. The percent identity then is calculated by the algorithm. Homologues will typically have one or more amino acid substitutions, deletions, and/or insertions as compared with the comparison Peptide, as the case may be.

The term "fusion protein" refers to a protein where one or more Peptides are recombinantly fused or chemically conjugated (including covalently and non-covalently) to a protein such as (but not limited to) an antibody or antibody fragment like an F_{ab} fragment or short chain Fv. The term "fusion protein" also refers to multimers (i.e. dimers, trimers, tetramers and higher multimers) of Peptides. Such multimers comprise homomeric multimers comprising one Peptide, heteromeric multimers comprising more than one Peptide, and heteromeric multimers comprising at least one Peptide and at least one other protein. Such multimers may be the result of hydrophobic, hydrophilic, ionic and/or covalent associations, bonds or links, may be formed by cross-links using linker molecules or may be linked indirectly by, for example, liposome formation

The term "peptide mimetic" or "mimetic" refers to biologically active compounds that mimic the biological activity of a peptide or a protein but are no longer peptidic in chemical nature, that is, they no longer contain any peptide bonds (that is, amide bonds between amino acids). Here, the term peptide mimetic is used in a broader sense to include molecules that are no longer completely peptidic in nature, such as pseudopeptides, semi-peptides and peptoids. Examples of peptide mimetics in this broader sense (where part of a peptide is replaced by a structure lacking peptide bonds) are described below. Whether completely or partially non-peptide, peptide mimetics according to this invention provide a spatial arrangement of reactive chemical moieties that closely resemble the three-dimensional arrangement of active groups in the Peptide on which the peptide mimetic is based. As a result of this similar active-site geometry, the peptide mimetic has effects on biological systems that are similar to the biological activity of the Peptide.

The peptide mimetics of this invention are preferably substantially similar in both three-dimensional shape and biological activity to the Peptides described herein. Examples of methods of structurally modifying a peptide known in the art to create a peptide mimetic include the inversion of backbone chiral centers leading to D-amino acid residue structures that may, particularly at the N-terminus, lead to enhanced stability for proteolytical degradation without adversely affecting activity. An example is given in the paper "Tritriated D-alal-Peptide T Binding", Smith C. S. et al., *Drug Development Res.,* 15, pp. 371-379 (1988). A second method is altering cyclic structure for stability, such as N to C interchain imides and lactames (Ede et al. in Smith and Rivier (Eds.) *Peptides: Chemistry and Biology*, Escom, Leiden (1991), pp. 268-270). An example of this is given in conformationally restricted thymopentin-like compounds, such as those disclosed in U.S. Pat. No. 4,457,489 (1985), Goldstein, G. et al., the disclosure of which is incorporated by reference herein in its entirety. A third method is to substitute peptide bonds in the Peptide by pseudopeptide bonds that confer resistance to proteolysis.

A number of pseudopeptide bonds have been described that in general do not affect peptide structure and biological activity. One example of this approach is to substitute retro-inverso pseudopeptide bonds ("Biologically active retroinverso analogues of thymopentin", Sisto A. et al in Rivier, J. E. and Marshall, G. R. (eds) *Peptides, Chemistry, Structure and Biology*, Escom, Leiden (1990), pp. 722-773) and Dalpozzo, et al. (1993), *Int. J. Peptide Protein Res.,* 41:561-566, incorporated herein by reference). According to this modification, the amino acid sequences of the peptides may be identical to the sequences of a Peptide described above, except that one or more of the peptide bonds are replaced by a retro-inverso pseudopeptide bond. Preferably the most N-terminal peptide bond is substituted, since such a substitution will confer resistance to proteolysis by exopeptidases acting on the N-terminus. Further modifications also can be made by replacing chemical groups of the amino acids with other chemical groups of similar structure. Another suitable pseudopeptide bond that is known to enhance stability to enzymatic cleavage with no or little loss of biological activity is the reduced isostere pseudopeptide bond (Couder, et al. (1993), *Int. J. Peptide Protein Res.,* 41:181-184, incorporated herein by reference in its entirety).

Thus, the amino acid sequences of these peptides may be identical to the sequences of a Peptide, except that one or more of the peptide bonds are replaced by an isostere pseudopeptide bond. Preferably the most N-terminal peptide bond is substituted, since such a substitution would confer resistance to proteolysis by exopeptidases acting on the N-terminus. The synthesis of peptides with one or more reduced isostere pseudopeptide bonds is known in the art (Couder, et al. (1993), cited above). Other examples include the introduction of ketomethylene or methylsulfide bonds to replace peptide bonds.

Peptoid derivatives of the Peptides represent another class of peptide mimetics that retain the important structural determinants for biological activity, yet eliminate the peptide bonds, thereby conferring resistance to proteolysis (Simon, et al., 1992, *Proc. Natl. Acad. Sci. USA,* 89:9367-9371, incorporated herein by reference in its entirety). Peptoids are oligomers of N-substituted glycines. A number of N-alkyl groups have been described, each corresponding to the side chain of a natural amino acid (Simon, et al. (1992), cited above). Some or all of the amino acids of the Peptides may be replaced with the N-substituted glycine corresponding to the replaced amino acid.

The term "peptide mimetic" or "mimetic" also includes reverse-D peptides and enantiomers as defined below.

The term "reverse-D peptide" refers to a biologically active protein or peptide consisting of D-amino acids arranged in a reverse order as compared to the L-amino acid sequence of a Peptide. Thus, the carboxy terminal residue of an L-amino acid Peptide becomes the amino terminal for the D-amino acid peptide and so forth. For example, the Peptide, FHDLKKHCIK (SEQ ID NO: 2), becomes $K_dI_dC_dH_dK_d$-$K_dL_dD_dH_dF_d$, where C_d, D_d, F_d, H_d, I_d, K_d, and L_d the D-amino acids corresponding to the L-amino acids, C, D, F, H, I, K, and L respectively.

The term "enantiomer" refers to a biologically active protein or peptide where one or more the L-amino acid residues in the amino acid sequence of a Peptide is replaced with the corresponding D-amino acid residue(s).

A "composition" as used herein, refers broadly to any composition containing a Peptide or amino acid sequence.

The composition may comprise a dry formulation, an aqueous solution, or a sterile composition. Compositions comprising one or more of the Peptides may be employed as hybridization probes. The probes may be stored in freeze-dried form and may be associated with a stabilizing agent such as a carbohydrate. In hybridizations, the probe may be deployed in an aqueous solution containing salts, e.g., NaCl, detergents, e.g., sodium dodecyl sulfate (SDS), and other components, e.g., Denhardt's solution, dry milk, salmon sperm DNA, etc.

Peptides and their fragments, variants, derivatives, homologues, fusion proteins and mimetics thereof encompassed by the embodiments can be prepared using methods known to those of skill in the art, such as recombinant DNA technology and protein synthesis.

A Peptide can be prepared using well known recombinant DNA technology methods such as those set forth in Sambrook et al. *Molecular Cloning: A Laboratory Manual,* 4th Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. [2012] and/or *Current Protocols in Molecular Biology*, Wiley Online Library, http://www.currentprotocols.com/WileyCDA/.

Another means to prepare a gene encoding a Peptide is to employ chemical synthesis using methods well known to the skilled artisan, such as those described by Engels et al., *Angew. Chem. Intl. Ed.,* 28:716-734 [1989]. These methods include, inter alia, the phosphotriester, phosphoramidite, and H-phosphonate methods for nucleic acid synthesis. A preferred method for such chemical synthesis is polymer-supported synthesis using standard phosphoramidite chemistry. Nucleic acids larger than about 100 nucleotides can be synthesized as several fragments using these methods. The fragments then can be ligated together to form the full length Peptide if necessary. Usually, the DNA fragment encoding the amino terminus of the protein will have an ATG, which encodes a methionine residue. This methionine may or may not be present on the mature form of the Peptide, depending on whether the protein produced in the host cell is designed to be secreted from that cell.

The gene, cDNA, or fragment thereof encoding the Peptide can be inserted into an appropriate expression or amplification vector using standard ligation techniques. The vector is typically selected to be functional in the particular host cell employed (i.e., the vector is compatible with the host cell machinery such that amplification of the gene and/or expression of the gene can occur). The gene, cDNA or fragment thereof encoding the Peptide may be amplified/expressed in prokaryotic, yeast, insect (baculovirus systems) and/or eukaryotic host cells. Selection of the host cell will depend in part on whether the Peptide is to be glycosylated and/or phosphorylated. If so, yeast, insect, or mammalian host cells are preferable.

Typically, the vectors used in any of the host cells will contain at least a 5' flanking sequence (also referred to as a promoter) and other regulatory elements as well, such as an enhancer(s), an origin of replication element, a transcriptional termination element, a complete intron sequence containing a donor and acceptor splice site, a signal peptide sequence, a ribosome binding site element, a polyadenylation sequence, a polylinker region for inserting the nucleic acid encoding the polypeptide to be expressed, and a selectable marker element. Each of these elements is discussed below. Optionally, the vector may contain a tag sequence, i.e., an oligonucleotide molecule located at the 5' or 3' end of the Peptide's coding sequence; the oligonucleotide molecule encodes polyHis (such as hexaHis (SEQ ID NO: 3)), or other tag such as FLAG, HA (hemaglutinin Influenza virus) or myc for which commercially available antibodies exist. This tag is typically fused to the polypeptide upon expression of the polypeptide, and can serve as means for affinity purification of the Peptide from the host cell. Affinity purification can be accomplished, for example, by column chromatography using antibodies against the tag as an affinity matrix. Optionally, the tag can subsequently be removed from the purified Peptide by various means such as using certain peptidases.

The human immunoglobulin hinge and Fc region could be fused at either the N-terminus or C-terminus of the Claimed Peptide by one skilled in the art. The subsequent Fc-fusion protein could be purified by use of a Protein A affinity column. Fc is known to exhibit a long pharmacokinetic half-life in vivo and proteins fused to Fc have been found to exhibit a substantially greater half-life in vivo than the unfused counterpart. Also, fusion to the Fc region allows for dimerization/multimerization of the molecule that may be useful for the bioactivity of some molecules.

The 5' flanking sequence may be homologous (i.e., from the same species and/or strain as the host cell), heterologous (i.e., from a species other than the host cell species or strain), hybrid (i.e., a combination of 5' flanking sequences from more than one source), synthetic, or it may be the native Peptide gene 5' flanking sequence. As such, the source of the 5' flanking sequence may be any unicellular prokaryotic or eukaryotic organism, any vertebrate or invertebrate organism, or any plant, provided that the 5' flanking sequence is functional in, and can be activated by, the host cell machinery.

The 5' flanking sequences useful in the vectors of the embodiments may be obtained by any of several methods well known in the art.

The origin of replication element is typically a part of prokaryotic expression vectors purchased commercially, and aids in the amplification of the vector in a host cell. Amplification of the vector to a certain copy number can, in some cases, be important for optimal expression of the Peptide. If the vector of choice does not contain an origin of replication site, one may be chemically synthesized based on a known sequence, and ligated into the vector. The transcription termination element is typically located 3' of the end of the coding sequence for the Peptide and serves to terminate transcription of the Peptide. Usually, the transcription termination element in prokaryotic cells is a G-C rich fragment followed by a poly T sequence. While the element may be cloned from a library or purchased commercially as part of a vector, it can also be readily synthesized using methods for nucleic acid synthesis such as those described above.

A selectable marker gene element encodes a protein necessary for the survival and growth of a host cell grown in a selective culture medium. Typical selection marker genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, tetracycline, or kanamycin for prokaryotic host cells, (b) complement auxotrophic deficiencies of the cell; or (c) supply critical nutrients not available from complex media. Preferred selectable markers are the kanamycin resistance gene, the ampicillin resistance gene, and the tetracycline resistance gene.

The ribosome binding element, commonly called the Shine-Dalgarno sequence (prokaryotes) or the Kozak sequence (eukaryotes), is usually necessary for translation initiation of mRNA. The element is typically located 3' to the promoter and 5' to the coding sequence of the Claimed Peptide to be synthesized. The Shine-Dalgarno sequence is varied but is typically a polypurine (i.e., having a high A-G content). Many Shine-Dalgarno sequences have been identified, each of which can be readily synthesized using methods set forth above and used in a prokaryotic vector.

In those cases where it is desirable for the Peptide to be secreted from the host cell, a signal sequence may be used to direct the Peptide out of the host cell where it is synthesized, and the carboxy-terminal part of the protein may be deleted in order to prevent membrane anchoring. Typically, the signal sequence is positioned in the coding region of the Peptide gene or cDNA, or directly at the 5' end of the Peptide gene coding region. Many signal sequences have been identified, and any of them that are functional in the selected host cell may be used in conjunction with the Peptide gene or cDNA. Therefore, the signal sequence may be homologous or heterologous to the Peptide gene or cDNA, and may be homologous or heterologous to the Peptide gene or cDNA. Additionally, the signal sequence may be chemically synthesized using methods set forth above. In most cases, secretion of the polypeptide from the host cell via the presence of a signal peptide will result in the removal of the amino terminal methionine from the polypeptide.

In many cases, transcription of the Peptide gene or cDNA is increased by the presence of one or more introns in the vector; this is particularly true where the Peptide is produced in eukaryotic host cells, especially mammalian host cells. The position of the intron with respect to the flanking sequence and the Peptide gene generally is important, as the intron must be transcribed to be effective. As such, where the Peptide gene inserted into the expression vector is a cDNA molecule, the preferred position for the intron is 3' to the transcription start site, and 5' to the polyA transcription termination sequence. Preferably for Peptide cDNA, the intron or introns will be located on one side or the other (i.e., 5' or 3') of the cDNA such that it does not interrupt this coding sequence. Any intron from any source, including any viral, prokaryotic and eukaryotic (plant or animal) organisms, may be used to practice this invention, provided that it is compatible with the host cell(s) into which it is inserted. Also included herein are synthetic introns. Optionally, more than one intron may be used in the vector.

Where one or more of the elements set forth above are not already present in the vector to be used, they may be individually obtained and ligated into the vector. Methods used for obtaining each of the elements are well known to the skilled artisan and are comparable to the methods set forth above (i.e., synthesis of the DNA, library screening, and the like).

The final vectors used to practice this invention may be constructed from starting vectors such as a commercially available vector. Such vectors may or may not contain some of the elements to be included in the completed vector. If none of the desired elements are present in the starting vector, each element may be individually ligated into the vector by cutting the vector with the appropriate restriction endonuclease(s) such that the ends of the element to be ligated in and the ends of the vector are compatible for ligation. In some cases, it may be necessary to blunt the ends to be ligated together in order to obtain a satisfactory ligation. Blunting is accomplished by first filling in "sticky ends" using Klenow DNA polymerase or T4 DNA polymerase in the presence of all four nucleotides. This procedure is well known in the art and is described for example in Sambrook et al., supra. Alternatively, two or more of the elements to be inserted into the vector may first be ligated together (if they are to be positioned adjacent to each other) and then ligated into the vector.

An additional method for constructing the vector is to conduct all ligations of the various elements simultaneously in one reaction mixture. Here, many nonsense or nonfunctional vectors will be generated due to improper ligation or insertion of the elements, however the functional vector may be identified and selected by restriction endonuclease digestion.

Preferred vectors for practicing this invention are those that are compatible with bacterial, insect, and mammalian host cells. Such vectors include, inter alia, pCRII, pCR3, and pcDNA3.1 (Invitrogen Company, San Diego, Calif.), pBSII (Stratagene Company, La Jolla, Calif.), pET15b (Novagen, Madison, Wis.), PGEX (Pharmacia Biotech, Piscataway, N.J.), pEGFP-N2 (Clontech, Palo Alto, Calif.), pETL (BlueBacII; Invitrogen), and pFastBacDual (Gibco/BRL, Grand Island, N.Y.).

After the vector has been constructed and a nucleic acid molecule encoding full length or truncated Peptide has been inserted into the proper site of the vector, the completed vector may be inserted into a suitable host cell for amplification and/or polypeptide expression. Host cells may be prokaryotic host cells (such as *E. coli*) or eukaryotic host cells (such as a yeast cell, an insect cell, or a vertebrate cell). The host cell, when cultured under appropriate conditions, can synthesize Peptide which can subsequently be collected from the culture medium (if the host cell secretes it into the medium) or directly from the host cell producing it (if it is not secreted).

After collection, the Peptide can be purified using methods such as molecular sieve chromatography, affinity chromatography, and the like. Selection of the host cell for Peptide production will depend in part on whether the Peptide is to be glycosylated or phosphorylated (in which case eukaryotic host cells are preferred), and the manner in which the host cell is able to fold the protein into its native tertiary structure (e.g., proper orientation of disulfide bridges, etc.) such that biologically active protein is prepared by the Peptide that has biological activity, the Peptide may be folded after synthesis using appropriate chemical conditions as discussed below. Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO), human embryonic kidney (HEK) 293, 293T cells, or 3T3 cells. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. Other suitable mammalian cell lines, are the monkey COS-1 and COS-7 cell lines, and the CV-1 cell line. Further exemplary mammalian host cells include primate cell lines and rodent cell lines, including transformed cell lines. Normal diploid cells, cell strains derived from in vitro culture of primary tissue, as well as primary explants, are also suitable. Candidate cells may be genotypically deficient in the selection gene, or may contain a dominantly acting selection gene. Other suitable mammalian cell lines include but are not limited to, mouse neuroblastoma N2A cells, HeLa, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines.

Similarly useful as host cells suitable for the embodiments described herein are bacterial cells. For example, the various strains of *E. coli* (e.g., HB101, DH5a, DH10, and MC1061) are well-known as host cells in the field of biotechnology. Various strains of *B. subtilis, Pseudomonas* spp., other *Bacillus* spp., *Streptomyces* spp., and the like may also be employed in this method. Many strains of yeast cells known to those skilled in the art also are available as host cells for expression of the polypeptides of the present invention.

Additionally, where desired, insect cell systems may be utilized in the methods described herein. Such systems are described for example in Kitts et al. (*Biotechniques,* 14:810-817 [1993]), Lucklow (*Curr. Opin. Biotecinol.,* 4:564-572

[1993]) and Lucklow et al. (*J. Virol.,* 67:4566-4579 [1993]). Preferred insect cells are Sf-9 and Hi5 (Invitrogen, Carlsbad, Calif.).

Insertion (also referred to as transformation or transfection) of the vector into the selected host cell may be accomplished using such methods as calcium chloride, electroporation, microinjection, lipofection, or the DEAE-dextran method. The method selected will in part be a function of the type of host cell to be used. These methods and other suitable methods are well known to the skilled artisan, and are set forth, for example, in Sambrook et al., supra.

The host cells containing the vector (i.e., transformed or transfected) may be cultured using standard media well known to the skilled artisan. The media will usually contain all nutrients necessary for the growth and survival of the cells. Suitable media for culturing *E. coli* cells are for example, Luria Broth (LB) and/or Terrific Broth (TB). Suitable media for culturing eukaryotic cells are RPMI 1640, MEM, DMEM, all of which may be supplemented with serum and/or growth factors as required by the particular cell line being cultured. A suitable medium for insect cultures is Grace's medium supplemented with yeastolate, lactalbumin hydrolysate, and/or fetal calf serum as necessary. Typically, an antibiotic or other compound useful for selective growth of the transformed cells only is added as a supplement to the media. The compound to be used will be dictated by the selectable marker element present on the plasmid with which the host cell was transformed. For example, where the selectable marker element is kanamycin resistance, the compound added to the culture medium will be kanamycin.

The amount of Peptide produced in the host cell can be evaluated using standard methods known in the art. Such methods include, without limitation, Western blot analysis, SDS polyacrylamide gel electrophoresis, non-denaturing gel electrophoresis, HPLC separation, mass spectroscopy, immunoprecipitation, and/or activity assays such as DNA binding gel shift assays. If the Peptide has been designed to be secreted from the host cells, the majority of the Peptide may be found in the cell culture medium. Proteins prepared in this way will typically not possess an amino terminal methionine, as it is removed during secretion from the cell. If however, the Peptide is not secreted from the host cells, it will be present in the cytoplasm and/or the nucleus (for eukaryotic host cells) or in the cytosol (for gram negative bacteria host cells) and may have an amino terminal methionine.

For Peptides situated in the host cell cytoplasm and/or nucleus, the host cells typically are first disrupted mechanically or with detergent to release the intra-cellular contents into a buffered solution. The Peptide can then be isolated from this solution.

Purification of Peptide from solution can be accomplished using a variety of techniques. If the protein has been synthesized such that it contains a tag such as hexaHistidine (e.g. Peptide/hexaHis) (SEQ ID NO: 3) or other small peptide such as FLAG (Sigma-Aldritch, St. Louis, Mich.) or calmodulin-binding peptide (Stratagene, La Jolla, Calif.) at either its carboxyl or amino terminus, it may essentially be purified in a one-step process by passing the solution through an affinity column where the column matrix has a high affinity for the tag or for the protein directly (i.e., a monoclonal antibody specifically recognizing the Peptide). For example, polyhistidine binds with great affinity and specificity to nickel, zinc and cobalt; thus immobilized metal ion affinity chromatography which employs a nickel-based affinity resin (as used in Qiagen's Q1Aexpress system or Invitrogen's Xpress System) or a cobalt-based affinity resin (as used in BD Biosciences-CLONTECH's Talon system) can be used for purification of Peptide/polyHis. (See, for example, *Current Protocols in Molecular Biology*).

Where the Peptide is prepared without a tag attached, and no antibodies are available, other well known procedures for purification can be used. Such procedures include, without limitation, ion exchange chromatography, hydroxyapatite chromatography, hydrophobic interaction chromatography, molecular sieve chromatography, HPLC, native gel electrophoresis in combination with gel elution, and preparative isoelectric focusing (Isoprime machine/technique, Hoefer Scientific). In some cases, two or more of these techniques may be combined to achieve increased purity.

If it is anticipated that the Peptide will be found primarily intracellularly, the intracellular material (including inclusion bodies for gram-negative bacteria) can be extracted from the host cell using any standard technique known to the skilled artisan. For example, the host cells can be lysed to release the contents of the periplasm/cytoplasm by French press, homogenization, and/or sonication followed by centrifugation. If the Peptide has formed inclusion bodies in the cytosol, the inclusion bodies can often bind to the inner and/or outer cellular membranes and thus will be found primarily in the pellet material after centrifugation. The pellet material then can be treated at pH extremes or with chaotropic agent such as a detergent, guanidine, guanidine derivatives, urea, or urea derivatives in the presence of a reducing agent such as dithiothreitol at alkaline pH or tris carboxyethyl phosphine at acid pH to release, break apart, and solubilize the inclusion bodies. The Peptide in its now soluble form can then be analyzed using gel electrophoresis, immunoprecipitation or the like. If it is desired to isolate the Peptide, isolation may be accomplished using standard methods such as those set forth below and in Marston et al. *Meth. Enz.,* 182:264-275 (1990).

In some cases, the Peptide may not be biologically active upon isolation. Various methods for refolding or converting the polypeptide to its tertiary structure and generating disulfide linkages, can be used to restore biological activity. Such methods include exposing the solubilized polypeptide to a pH usually above 7 and in the presence of a particular concentration of a chaotrope. The selection of chaotrope is very similar to the choices used for inclusion body solubilization but usually at a lower concentration and is not necessarily the same chaotrope as used for the solubilization. In most cases the refolding/oxidation solution will also contain a reducing agent or the reducing agent plus its, oxidized form in a specific ratio to generate a particular redox potential allowing for disulfide shuffling to occur in the formation of the protein's cysteine bridge(s). Some of the commonly used redox couples include cysteine/cystamine, glutathione (GSH)/dithiobis GSH, cupric chloride, dithiothreitol(DTT)/dithiane DTT, 2-mercaptoethanol(bME)/dithio-b(ME). In many instances a cosolvent is necessary to increase the efficiency of the refolding and the more common reagents used for this purpose include glycerol, polyethylene glycol of various molecular weights, and arginine.

If Peptide inclusion bodies are not formed to a significant degree in the host cell, the Peptide will be found primarily in the supernatant after centrifugation of the cell homogenate, and the Peptide can be isolated from the supernatant using methods such as those set forth below.

In those situations where it is preferable to partially or completely isolate the Peptide, purification can be accomplished using standard methods well known to the skilled artisan. Such methods include, without limitation, separation by electrophoresis followed by electroelution, various types of chromatography (immunoaffinity, molecular sieve, and/or

ion exchange), and/or high pressure liquid chromatography. In some cases, it may be preferable to use more than one of these methods for complete purification.

In addition to preparing and purifying the Peptide using recombinant DNA techniques, the Peptide and their fragments, variants, homologues, fusion proteins, peptide mimetics, and derivatives may be prepared by chemical synthesis methods (such as solid phase peptide synthesis) using techniques known in the art, such as those set forth by Merrifield et al., *J. Am. Chem. Soc.,* 85:2149 [1963], Houghten et al. *Proc Natl Acad. Sci. USA,* 82:5132 [1985], and Stewart and Young, *Solid Phase Peptide Synthesis*, Pierce Chemical Co., Rockford, Ill. [1984]. Such polypeptides may be synthesized with or without a methionine on the amino terminus. Chemically synthesized Peptides may be oxidized using methods set forth in these references to form disulfide bridges. The Peptides are expected to have biological activity comparable to Peptides produced recombinantly and thus may be used interchangeably with recombinant Peptide.

Chemically modified Peptide compositions in which the Peptide is linked to a polymer also are included within the scope of the embodiments. The polymer selected is typically water soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. The polymer selected may be modified to have a single reactive group, such as an active ester for acylation or an aldehyde for alkylation, so that the degree of polymerization may be controlled as provided for in the present methods. The polymer may be of any molecular weight, and may be branched or unbranched. Included within the scope of Peptide polymers is a mixture of polymers.

In some cases, it may be desirable to prepare nucleic acid and/or amino acid variants of the Peptides. Nucleic acid variants may be produced using site directed mutagenesis, PCR amplification, or other appropriate methods, where the primer(s) have the desired point mutations (see Sambrook et al., supra, and *Current Protocols in Molecular Biology*, supra, for descriptions of mutagenesis techniques). Chemical synthesis using methods described by Engels et al., supra, may also be used to prepare such variants. Other methods known to the skilled artisan may be used as well.

Preferred nucleic acid variants are those containing nucleotide substitutions accounting for codon preference in the host cell that is to be used to produce the Peptide. Such codon optimization can be determined via computer algorithers which incorporate codon frequency tables such as Ecohigh.cod for codon preference of highly expressed bacterial genes as provided by the University of Wisconsin Package Version 9.0, Genetics Computer Group, Madison, Wis. Other useful codon frequency tables include Celegans_high.cod, Celegans_low.cod, *Drosophila*_high.cod, Human_high.cod, Maize_high.cod, and Yeast_high.cod. Other preferred variants are those encoding conservative amino acid changes as described above (e.g., wherein the charge or polarity of the naturally occurring amino acid side chain is not altered substantially by substitution with a different amino acid) as compared to wild type, and/or those designed to either generate a novel glycosylation and/or phosphorylation site(s), or those designed to delete an existing glycosylation and/or phosphorylation site(s).

The Peptides and their fragments, homologs, variants, fusion proteins, peptide mimetics, derivatives and salts thereof also can be made using conventional peptide synthesis techniques known to the skilled artisan. These techniques include chemical coupling methods (cf. Wunsch, E: "Methoden der organischen Chemie", Volume 15, Band 1+2, *Synthese von Peptiden, thime Verlag*, Stuttgart (1974), and Barrany, G.; Marrifield, R. B.: "The Peptides," eds. E. Gross, J. Meienhofer, Volume 2, Chapter 1, pp. 1-284, Academic Press (1980)), enzymatic coupling methods (cf. Widmer, F. Johansen, J. T., *Carlsberg Res. Commun*., Vol. 44, pp. 3746 (1979); Kullmann, W.: "Enzymatic Peptide Synthesis", CRC Press Inc. Boca Raton, Fla. (1987); and Widmer, F., Johansen, J. T. in "Synthetic Peptides in Biology and Medicines," eds. Alitalo, K., Partanen, P., Vatieri, A., pp. 79-86, Elsevier, Amsterdam (1985)), or a combination of chemical and enzymatic methods if this is advantageous for the process design and economy. Using the guidelines provided herein, those skilled in the art are capable of varying the peptide sequence of the Peptide to make a homologue having the same or similar biological activity (bioactivity) as the original Peptide.

Advantages exist for using a mimetic of a given Peptide rather than the peptide itself. In general, peptide mimetics are more bioavailable, have a longer duration of action and can be cheaper to produce than the native proteins and peptides. Thus the Peptides described herein have utility in the development of such small chemical compounds with similar biological activities and therefore with similar therapeutic utilities. Peptide mimetics of Peptides can be developed using combinatorial chemistry techniques and other techniques known in the art (see e.g. Proceedings of the 20th European Peptide Symposium, ed. G. Jung, E. Bayer, pp. 289-336, and references therein).

Examples of methods known in the art for structurally modifying a Peptide to create a peptide mimetic include the inversion of backbone chiral centers leading to D-amino acid residue structures that may, particularly at the N-terminus, lead to enhanced stability for proteolytical degradation without adversely affecting activity. An example is provided in the paper "Tritriated D-alal-Peptide T Binding", Smith C. S. et al., *Drug Development Res.* 15, pp. 371-379 (1988).

A second method includes altering cyclic structure for stability, such as N to C interchain imides and lactames (Ede et al. in Smith and Rivier (Eds.) "Peptides: Chemistry and Biology", Escom, Leiden (1991), pp. 268-270). An example of this is given in conformationally restricted thymopentin-like compounds, such as those disclosed in U.S. Pat. No. 4,457,489 (1985), Goldstein, G. et al., the disclosure of which is incorporated by reference herein in its entirety.

A third method includes substituting peptide bonds in the Peptide by pseudopeptide bonds that confer resistance to proteolysis. A number of pseudopeptide bonds have been described that in general do not affect peptide structure and biological activity. One example of this approach is to substitute retro-inverso pseudopeptide bonds ("Biologically active rettoinverso analogues of thymopentin", Sisto A. et al in Rivier, J. E. and Marshall, G. R. (eds) "Peptides, Chemistry, Structure and Biology", Escom, Leiden (1990), pp. 722-773) and Dalpozzo, et al. (1993), *Int. J. Peptide Protein Res.,* 41:561-566, incorporated herein by reference). According to this modification, the amino acid sequences of the peptides may be identical to the sequences of the Peptides described above, except that one or more of the peptide bonds are replaced by a retro-inverso pseudopeptide bond. Preferably the most N-terminal peptide bond is substituted, since such a substitution will confer resistance to proteolysis by exopeptidases acting on the N-terminus.

The synthesis of peptides with one or more reduced retto-inverso pseudopeptide bonds is known in the art (Sisto (1990) and Dalpozzo, et al. (1993), cited above). Thus, peptide bonds can be replaced by non-peptide bonds that allow the peptide mimetic to adopt a similar structure, and therefore biological activity, to the original peptide. Further modifications also

can be made by replacing chemical groups of the amino acids with other chemical groups of similar structure. Another suitable pseudopeptide bond that is known to enhance stability to enzymatic cleavage with no or little loss of biological activity is the reduced isostere pseudopeptide bond is a (Couder, et al. (1993), *Int. J. Peptide Protein Res.,* 41:181-184,). Thus, the amino acid sequences of these peptides may be identical to the sequences of a Peptide, except that one or more of the peptide bonds are replaced by an isostere pseudopeptide bond. Preferably the most N-terminal peptide bond is substituted, since such a substitution would confer resistance to proteolysis by exopeptidases acting on the N-terminus. The synthesis of peptides with one or more reduced isostere pseudopeptide bonds is known in the art (Couder, et al. (1993), cited above). Other examples include the introduction of ketomethylene or methylsulfide bonds to replace peptide bonds. A person having ordinary skill in the art will be capable of preparing a suitable peptide mimetic of the Peptides using the techniques disclosed herein.

Peptoid derivatives of Peptides represent another class of peptide mimetics that retain the important structural determinants for biological activity, yet eliminate the peptide bonds, thereby conferring resistance to proteolysis (Simon, et al., 1992, *Proc. Natl. Acad. Sci. USA,* 89:9367-9371. Peptoids are oligomers of N-substituted glycines. A number of N-alkyl groups have been described, each corresponding to the side chain of a natural amino acid (Simon, et al. (1992), supra). Some or all of the amino acids of the Peptide are replaced with the N-substituted glycine corresponding to the replaced amino acid.

The development of peptide mimetics can be aided by determining the tertiary structure of the original Peptide by NMR spectroscopy, crystallography and/or computer-aided molecular modeling. These techniques aid in the development of novel compositions of higher potency and/or greater bioavailability and/or greater stability than the original peptide (Dean (1994), *BioEssays,* 16: 683-687; Cohen and Shatzmiller (1993), *J. Mol. Graph.,* 11: 166-173; Wiley and Rich (1993), *Med. Res. Rev.,* 13: 327-384; Moore (1994), *Trends Pharmacol. Sci.,* 15: 124-129; Hruby (1993), *Biopolymers,* 33: 1073-1082; Bugg et al. (1993), *Sci. Am.,* 269: 92-98).

Once a potential peptide mimetic compound is identified, it may be synthesized and assayed using the methods outlined in the examples below to assess its activity. The peptide mimetic compounds obtained by the above methods, having the biological activity of the Peptide and similar three-dimensional structure, are encompassed by this invention. It will be readily apparent to one skilled in the art that a peptide mimetic can be generated from any of the Peptides bearing one or more of the modifications described above. It will furthermore be apparent that the peptide mimetics of the embodiments can be further used for the development of even more potent non-peptidic compounds, in addition to their utility as therapeutic compounds.

A number of organizations exist today that are capable of synthesizing the Peptides described herein. For example, given the sequence of one of the Peptides, the organization can synthesize the Peptide and forward the synthesized Peptide with accompanying documentation and proof of the identity of the Peptide.

The embodiments also encompass the use of Peptides and their corresponding nucleic acid molecules for assays to test, either qualitatively or quantitatively, for the presence of Peptides, Peptide DNA, or corresponding RNA in mammalian tissue or bodily fluid samples. A Peptide and its corresponding nucleic acid molecules may have use in the preparation in such assays, whether or not the Peptide or the encoded Peptide DNA show biological activity. Peptide nucleic acid sequences may be a useful source of hybridization probes to test, either qualitatively or quantitatively, for the presence of Peptide DNA or corresponding RNA in mammalian tissue or bodily fluid samples. A Peptide that is not in itself biologically active may be useful for preparing antibodies that recognize and/or bind to Peptides. Such antibodies may be prepared using standard methods. Thus, antibodies that react with or bind to a Peptide, as well as short chain antibody fragments and other reactive fragments of such antibodies, also are contemplated as within the scope of the present invention. The antibodies may be polyclonal, monoclonal, recombinant, chimeric, single-chain and/or bispecific. Typically, the antibody or fragment thereof will either be of human origin, or will be humanized, i.e., prepared so as to prevent or minimize an immune reaction to the antibody when administered to a patient. Preferred antibodies are human antibodies, either polyclonal or monoclonal. The antibody fragment may be any fragment that is reactive with a Peptide of the present invention, such as F_{ab}, F_{ab}, etc. Also provided by this invention are the hybridomas generated by presenting any Peptide as an antigen to a selected mammal, followed by fusing cells (e.g., spleen cells) of the mammal with certain cancer cells to create immortalized cell lines by known techniques. The methods employed to generate such cell lines and antibodies directed against all or portions of a Peptide are also encompassed by the embodiments.

The antibodies may further be used for in vivo and in vitro diagnostic or research purposes, such as in labeled form to detect the presence of a Peptide in a body fluid or cell sample.

The embodiments also encompass the use of one or more of the Peptides as calibration standards in assays that test, either qualitatively or quantitatively, for the presence of Peptide, Peptide DNA or corresponding RNA in mammalian tissue or bodily fluid samples.

The embodiments also include methods of treating conditions requiring removal of cells, such as benign and malignant tumors, glandular (e.g. prostate) hyperplasia, hypertrophic inflammatory masses, unwanted facial hair, warts, and unwanted fatty tissue, or the inhibition or prevention of unwanted cellular proliferation, such as stenosis of a stent. Such a method comprises administering to a mammal in need, or coating a device such as a stent with, a therapeutically effective amount of Peptide. The condition can be, for example, tumors of lung, breast, stomach, pancreas, prostate, bladder, bone, ovary, skin, kidney, sinus, colon, intestine, stomach, rectum, esophagus, blood, brain and its coverings, spinal cord and its coverings, muscle, connective tissue, adrenal, parathyroid, thyroid, uterus, testis, pituitary, reproductive organs, liver, gall bladder, eye, ear, nose, throat, tonsils, mouth, lymph nodes and lymphoid system, and other organs.

As used herein, the phrase "malignant tumor" is intended to encompass all forms of human carcinomas, sarcomas and melanomas which occur in the poorly differentiated, moderately differentiated, and well-differentiated forms.

The embodiments satisfy a need in the art for treatments that can remove benign or localized malignant tumors with less risk and fewer of the undesirable side effects of surgery. A method for removing benign or localized malignant tumors in surgically hazardous areas such as in deep locations in the body (e.g., brain, heart, lungs, and others) is particularly needed.

The method of treating conditions where cells must be removed can be used in conjunction with conventional methods of treating such conditions, such as surgical excision,

chemotherapy, and radiation. A Peptide can be administered before, during, or after such conventional treatments.

The condition to be treated can also be a hyperplasia, hypertrophy, or overgrowth of a tissue selected from the group consisting of lung, breast, stomach, pancreas, prostate, bladder, bone, ovary, skin, kidney, sinus, colon, intestine, stomach, rectum, esophagus, blood, brain and its coverings, spinal cord and its coverings, muscle, connective tissue, adrenal, parathyroid, thyroid, uterus, testis, pituitary, reproductive organs, liver, gall bladder, eye, ear, nose, throat, tonsils, mouth, and lymph nodes and lymphoid system.

Other conditions that can be treated using the methods described herein include virally, bacterially, or parasitically altered tissue selected from the group consisting of lung, breast, stomach, pancreas, prostate, bladder, bone, ovary, skin, kidney, sinus, colon, intestine, stomach, rectum, esophagus, blood, brain and its coverings, spinal cord and its coverings, muscle, connective tissue, adrenal, parathyroid, thyroid, uterus, testis, pituitary, reproductive organs, liver, gall bladder, eye, ear, nose, throat, tonsils, mouth, and lymph nodes and lymphoid system.

The condition to be treated can also be a malformation or disorder of a tissue selected from the group consisting of lung, breast, stomach, pancreas, prostate, bladder, bone, ovary, skin, kidney, sinus, colon, intestine, stomach, rectum, esophagus, blood, brain and its coverings, spinal cord and its coverings, muscle, connective tissue, adrenal, parathyroid, thyroid, uterus, testis, pituitary, reproductive organs, liver, gall bladder, eye, ear, nose, throat, tonsils, mouth, and lymph nodes and lymphoid system.

In particular, the condition to be treated can be tonsillar hypertrophy, prostatic hyperplasia, psoriasis, eczema, dermatoses or hemorrhoids. The condition to be treated can be a vascular disease, such as atherosclerosis or arteriosclerosis, or a vascular disorder, such as varicose veins, stenosis or restenosis of an artery or a stent. The condition to be treated can also be a cosmetic modification to a tissue, such as skin, eye, ear, nose, throat, mouth, muscle, connective tissue, hair, or breast tissue.

Therapeutic compositions of Peptides also are contemplated herein. Such compositions may comprise a therapeutically effective amount of a Peptide in admixture with a pharmaceutically acceptable carrier. The carrier material may be water for injection, preferably supplemented with other materials common in solutions for administration to mammals. Typically, a Peptide for therapeutic use will be administered in the form of a composition comprising at least one purified Peptide in conjunction with one or more physiologically acceptable carriers, excipients, or diluents. Phosphate buffered saline or saline mixed with serum albumin are exemplary appropriate carriers. Preferably, the product is formulated as a lyophilizate using appropriate excipients (e.g., sucrose). Other standard carriers, diluents, and excipients may be included as desired. Compositions also may comprise buffers known to those having ordinary skill in the art with an appropriate range of pH values, including Tris buffer of about pH 7.0-8.5, or acetate buffer of about pH 4.0-5.5, which may further include sorbitol or a suitable substitute therefor. Examples of aqueous vehicles and diluents include sodium chloride for injection, Ringers solution for injection, isotonic dextrose for injection, sterile water for injection, dextrose and lactated Ringers solution for injection. The carrier or diluent may include antimicrobial agents in bacteriostatic or fungistatic concentrations such as phenols or cresols, mercurials, benzyl alcohol, chlorobutanol, methyl and propyl p-hydroxybenzoic acid esters, thimerosal, benzalkonium chloride and benzethonium chloride. Isotonic agents include sodium chloride and dextrose, buffers such as phosphate and citrate, and antioxidants such as sodium bisulfate.

The use of a Peptide conjugated or linked or bound to an antibody, antibody fragment, antibody-like molecule, or a molecule with a high affinity to a specific tumor marker, such as a cellular receptor, signal peptide or over-expressed enzyme, for targeting to the unwanted cellular elements also is encompassed by the scope of the invention. The antibody, antibody fragment, antibody-like molecule, or molecule with a high affinity to a specific tumor marker may be used to target the Peptide conjugate to a specific cellular or tissue target. For example, a tumor with a distinctive surface antigen or expressed antigen can be targeted by the antibody, antibody fragment, or antibody-like binding molecule and the tumor cells can be killed by the Claimed Peptide. Such an approach using antibody targeting has the anticipated advantages of decreasing dosage, increasing the likelihood of binding to and uptake by the target cells, and increased usefulness for targeting and treating metastatic tumors and microscopic sized tumors.

The embodiments also encompass the use of a Peptide conjugated or linked or bound to a protein or other molecule to form a composition that, upon cleavage at or near the site(s) of the tumor or other unwanted cells by a tumor- or site-specific enzyme or protease or by an antibody conjugate that targets tumor or other unwanted cells, releases the Peptide at or near the site(s) of the tumor or other unwanted cells

The embodiments also encompass the use of a Peptide conjugated or linked or bound to a protein or other molecule to form a composition that releases the Peptide or some biologically active fragment of the Peptide upon exposure of the tissue to be treated to light (as in laser therapies or other photo-dynamic or photo-activated therapy), other forms of electro-magnetic radiation such as infra-red radiation, ultraviolet radiation, x-ray or gamma ray radiation, localized heat, alpha or beta radiation, ultrasonic emissions, or other sources of localized energy.

The Peptides may be employed alone, together, or in combination with other pharmaceutical compositions, such as cytokines, growth factors, antibiotics, apoptotis-inducing agents, anti-inflammatories, and/or chemotherapeutic agents as is appropriate for the indication being treated.

The embodiments also encompass therapeutic compositions of Peptides employing dendrimers, fullerenes, and other synthetic molecules, polymers and macromolecules where the Peptide and/or its corresponding DNA molecule is conjugated with, attached to or enclosed in the molecule, polymer or macromolecule, either by itself or in conjunction with other species of molecule such as a tumor-specific marker. For example, U.S. Pat. No. 5,714,166, Bioactive and/or Targeted Dendimer Conjugates, provides a method of preparing and using, inter alia, dendritic polymer conjugates composed of at least one dendrimer with a target director(s) and at least one bioactive agent conjugated to it. The disclosure of U.S. Pat. No. 5,714,166 is incorporated by reference herein in its entirety.

The embodiments also encompass therapeutic compositions of Peptides and/or genes and drug delivery vehicles such as lipid emulsions, micelle polymers, polymer microspheres, electroactive polymers, hydrogels and liposomes.

The use of Peptides or related genes or gene equivalents transferred to the unwanted cells also is encompassed by the invention. Overexpression of the Peptide within the tumor can be used to induce the cells in the tumor to die and thus reduce the tumor cell population. The gene or gene equivalent transfer of Peptide to treat the unwanted cellular elements is anticipated to have the advantage of requiring less dosage,

and of being passed on to the cellular progeny of the targeted cellular elements, thus necessitating less frequent therapy, and less total therapy. This invention also encompasses the transfer of genes that code for a fusion protein containing a Peptide to the unwanted cells or neighboring cells where, following the expression of the gene and the production and/or secretion of the fusion protein, the fusion protein is cleaved either by native enzymes or proteases or by a prodrug to release the Peptide in, at or near the unwanted cells.

The use of cloned recombinant Peptide-antibody conjugates; cloned recombinant Peptide-antibody fragment conjugates; and cloned recombinant Peptide-antibody-like protein conjugates is also encompassed by the embodiments. One of the advantages of a cloned Peptide combined with targeting conjugate (such as an antibody, antibody fragment, antibody-like molecule, or a molecule with a high affinity to a cancer-specific receptor or other tumor marker) is that such a molecule combines the targeting advantages described above in addition to advantages for manufacturing and standardized production of the cloned conjugated molecule.

The embodiments also encompass the use of therapeutic compositions of Peptide or genes or gene equivalents coding for Peptides as a component of the coating of a medical device such as a stent in order to remove, inhibit or prevent unwanted cellular proliferation or accumulation.

Solid dosage forms for oral administration include but are not limited to, capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compound is admixed with at least one of the following: (a) one or more inert excipients (or carrier), such as sodium citrate or dicalcium phosphate; (b) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and silicic acid; (c) binders, such as carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidone, sucrose and *acacia*; (d) humectants, such as glycerol; (e) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain complex silicates, and sodium carbonate; (f) solution retarders, such as paraffin; (g) absorption accelerators, such as quaternary ammonium compounds; (h) wetting agents, such as acetyl alcohol and glycerol monostearate; (i) adsorbents, such as kaolin and bentonite; and (j) lubricants, such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, or mixtures thereof. For capsules, tablets, and pills, the dosage forms may also comprise buffering agents.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, and elixirs. In addition to the active compounds, the liquid dosage forms may comprise inert diluents commonly used in the art, such as water or other solvents, solubilizing agents, and emulsifiers. Exemplary emulsifiers are ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propyleneglycol, 1,3-butyleneglycol, dimethylformamide, oils, such as cottonseed oil, groundnut oil, corn germ oil, olive oil, castor oil, and sesame oil, glycerol, tetrahydrofurfuryl alcohol, polyethyleneglycols, fatty acid esters of sorbitan, or mixtures of these substances, and the like.

Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents.

Preparations for parenteral administration include sterile solutions ready for injection, sterile dry insoluble products ready to be combined with a vehicle or diluent just prior to use and sterile emulsions. The solutions may be either aqueous or nonaqueous, and formulated for delivery by injection, infusion, or using implantable pumps. Injection devices for use in administering preparations include pre-filled syringes, dual chamber syringes and injector pens. Needle guide systems employed with an imaging system such as endoscopy, ultrasound imaging, MRI, or other remote imaging, scanning or sensing technology may be used to administer the preparation to a local site requiring treatment.

Actual dosage levels of active ingredients in the compositions described herein may be varied to obtain an amount of Peptide that is effective to obtain a desired therapeutic response for a particular composition and method of administration. The selected dosage level therefore depends upon the desired therapeutic effect, the route of administration, the site, nature, and size of the tumor or growth to be treated, the desired duration of treatment, and other factors.

With mammals, including humans, the effective amounts can be administered on the basis of body surface area. The interrelationship of dosages for animals of various sizes, species and humans (based on mg/M2 of body surface) is described by E. J. Freireich et al., *Cancer Chemother. Rep.,* 50 (4):219 (1966). Body surface area may be approximately determined from the height and weight of an individual (see e.g., Scientific Tables, Geigy Pharmaceuticals, Ardsley, N.Y. pp. 537-538 (1970)).

The total dose of the Peptide administered to a host may be in single or divided doses and may be given through a single dose, daily doses, or repeat administration of doses at intervals of days, weeks, months or years until the desired effect is achieved. Dosage unit compositions may contain such amounts of such submultiples thereof as may be used to make up the dose. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the body weight, general health, sex, diet, time and route of administration, potency of the administered drug, rates of absorption and excretion, combination with other drugs and the severity of the particular disease being treated.

A method of administering a Peptide composition according to the embodiments includes, but is not limited to, administering the compounds intramuscularly, orally, intravenously, intraperitoneally, intracerebrally (intraparenchymally), intracerebroventricularly, intratumorally, intraprostatically, intralesionally, intradermally, intrathecally, intranasally, intraocularly, intraarterially, topically, transdermally, via an aerosol, infusion, bolus injection, implantation device, sustained release system etc.

Another method of administering a Peptide is by a transdermal or transcutaneous route. One example of such an embodiment is the use of a patch. In particular, a patch can be prepared with a fine suspension of Peptide in, for example, dimethylsulfoxide (DMSO), or a mixture of DMSO with cottonseed oil and brought into contact with the skin of the tumor carrying mammals away from the tumor location site inside a skin pouch. Other mediums or mixtures thereof with other solvents and solid supports would work equally as well. The patch can contain the Peptide compound in the form of a solution or a suspension. The patch can then be applied to the skin of the patient, for example, by means of inserting it into a skin pouch of the patient formed by folding and holding the skin together by means of stitches, clips or other holding devices. This pouch should be employed in such a manner so that continuous contact with the skin is assured without the interference of the mammal. Besides using a skin pouch, any device can be used which ensures the firm placement of the patch in contact with the skin. For instance, an adhesive bandage could be used to hold the patch in place on the skin.

The Peptide may be administered in a sustained release formulation or preparation. Suitable examples of sustained-release preparations include semipermeable polymer matrices in the form of shaped articles, e.g. films, or microcapsules. Sustained release matrices include polyesters, hydrogels, polylactides (U.S. Pat. No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., Biopolymers, 22: 547-556 [1983]), poly (2-hydroxyethyl-methacrylate) (Langer et al., *J. Biomed. Mater. Res.,* 15: 167-277 [1981] and Langer, Chem. Tech., 12: 98-105 [1982]), ethylene vinyl acetate (Langer et al., supra) or poly-D(−)-3-hydroxybutyric acid (EP 133,988). Sustained-release compositions also may include liposomes, which can be prepared by any of several methods known in the art (e.g., Eppstein et al., *Proc. Natl. Acad. Sci. USA,* 82: 3688-3692 [1985]; EP 36,676; EP 88,046; and EP 143,949).

Another method of administering a Peptide of the embodiments is by direct or indirect infusion of Peptide into the tumor or other tissue to be treated. One example of such an embodiment is the direct injection of the Peptide into the tumor or other tissue to be treated. The treatment may consist of a single injection, multiple injections on one occasion or a series of injections over a period of hours, days or months with the regression or destruction of the tumor or other tissue to be treated being monitored by means of biopsy, imaging or other methods of monitoring tissue growth. The injection into the tumor or other tissue to be treated may be by a device inserted into an orifice such as the nose, mouth, ear, vagina, rectum or urethra or through an incision in order to reach the tumor or tissue in vivo and may performed in conjunction with an imaging or optical system such as ultrasound or fibre optic scope in order to identify the appropriate site for the injection(s). Another example of such an embodiment is the use of a device that can provide a constant infusion of Peptide to the tissue over time.

Another method of administering a Peptide of the embodiments is in conjunction with a surgical or similar procedure employed to physically excise, ablate or otherwise kill or destroy tumor or other tissue or cellular elements required or desired to be removed or destroyed wherein a Peptide of the embodiments is administered to the immediate area(s) surrounding the area(s) where the tumor or other tissue was removed in order to destroy or impede the growth of any tumor cells or other cellular elements not removed or destroyed by the procedure

Another method of administering a Peptide of the embodiments is by implantation of a device within the tumor or other tissue to be treated. One example of such an embodiment is the implantation of a wafer containing a Peptide in the tumor or other tissue to be treated. The wafer releases a therapeutic dose of the Peptide into the tissue over time. Alternatively or additionally, the composition may be administered locally via implantation into the affected area of a membrane, sponge, or other appropriate material on to which the Peptide has been absorbed, and preferably a bioresorbable membrane, sponge, or other appropriate material that will biodegrade over time in the body. Where an implantation device is used, the device may be implanted into any suitable tissue or organ, and delivery of the Peptide may be directly through the device via bolus, or via continuous administration, or via catheter using continuous infusion.

An alternative method of administration is to introduce one or more copies of a Peptide-encoding gene into the cell being targeted and, if necessary, inducing the copy(ies) of the gene to begin producing the Peptide intracellularly. One manner in which gene therapy can be applied is to use a gene that codes for the Peptide (or a fragment, variant, homologue or derivative thereof)) which may be operably linked to a constitutive or inducible promoter to form a gene therapy DNA construct. Other components of the gene therapy DNA construct may optionally include, as required, DNA molecules designed for site-specific integration (e.g., endogenous flanking sequences useful for homologous recombination), tissue-specific promoter, enhancer(s) or silencer(s), DNA molecules capable of providing a selective advantage over the parent cell, DNA molecules useful as labels to identify transformed cells, negative selection systems, cell specific binding agents (as, for example, for cell targeting) cell-specific internalization factors, and transcription factors to enhance expression by a vector as well as factors to enable vector manufacture.

Methods of gene delivery to a cell or tissue in vivo or ex vivo include (but are not limited to) direct injection of bare DNA, ballistic methods, liposome-mediated transfer, receptor-mediated transfer (ligand-DNA complex), electroporation, and calcium phosphate precipitation. See U.S. Pat. No. 4,970,154, WO 96/40958, U.S. Pat. No. 5,679,559, U.S. Pat. No. 5,676,954, and U.S. Pat. No. 5,593,875, the disclosures of each of which are incorporated by reference herein in their entirety. They also include use of a viral vector such as a retrovirus, adenovirus, adeno-associated virus, pox virus, lentivirus, papilloma virus or herpes simplex virus, use of a DNA-protein conjugate and use of a liposome. The use of gene therapy vectors is described, for example, in U.S. Pat. No. 5,672,344, U.S. Pat. No. 5,399,346, U.S. Pat. No. 5,631,236, and U.S. Pat. No. 5,635,399, the disclosures of each of which are incorporated by reference herein in their entirety.

The gene coding for the Peptide may be delivered through implanting into patients certain cells that have been genetically engineered ex vivo, using methods such as those described herein, to express and secrete the Peptide or fragments, variants, homologues, or derivatives thereof. Such cells may be animal or human cells, and may be derived from the patient's own tissue or from another source, either human or non-human. Optionally, the cells may be immortalized or be stem cells. However, in order to decrease the chance of an immunological response, it is preferred that the cells be encapsulated to avoid infiltration of surrounding tissues. The encapsulation materials are typically biocompatible, semipermeable polymeric enclosures or membranes that allow release of the protein product(s) but prevent destruction of the cells by the patient's immune system or by other detrimental factors from the surrounding tissues. Methods used for membrane encapsulation of cells are familiar to the skilled artisan, and preparation of encapsulated cells and their implantation in patients may be accomplished without undue experimentation. See, e.g., U.S. Pat. Nos. 4,892,538; 5,011,472; and 5,106,627, the disclosures of each of which are incorporated by reference herein in their entirety. A system for encapsulating living cells is described in PCT WO 91/10425. Techniques for formulating a variety of other sustained or controlled delivery means, such as liposome carriers, bioerodible particles or beads, are also known to those in the art, and are described, for example, in U.S. Pat. No. 5,653,975, the disclosure of which is incorporated by reference herein in their entirety. The cells, with or without encapsulation, may be implanted into suitable body tissues or organs of the patient.

The following examples are provided to illustrate the embodiments. It should be understood, however, that the embodiments are not to be limited to the specific conditions or details described in these examples. Throughout the specification, any and all references to a publicly available document, including a U.S. patent, are specifically incorporated by reference.

EXAMPLE 1

The purpose of this example was to determine the effect of peptide NYMAP1385 (SEQ ID NO. 1) on tissue at sites of injection.

After open laparotomy under general anesthesia, 8 normal 3 month old Sprague-Dawley male rats were injected in liver and in prostate, and in extremity skeletal muscle, with peptide NYMAP1385 in saline in quantities of 100 to 400 μL at a concentration of 1 mg/mL delivered from plastic syringes through stainless steel 26 gauge needles.

The animals were observed for 72 hours and painlessly sacrificed at 72 hours. The individual foci of infiltration were excised, fixed in 10% formalin, embedded in paraffin, and stained and examined by standard histopathological methods. For each animal the entire prostate gland was embedded and sectioned. All stained sections were examined histologically and measured. For each prostate at least 4 histological sections were examined, and for each histological section two cross-sectional diameters (D) at 90° from each other were measured (total of ≥4 measurements per prostate). The mean diameter from these measurements for each prostate was used to estimate volume according to

$$V=4/3*\pi(D/2)^3$$

Control rats received saline alone, or no injection, or other inactive peptides.

Results: Injection of NTP peptide NYMAP1385 produced variable amounts of apoptosis and necrosis of tissue at the injection sites. The cell loss was most evident in prostate and liver at the sites where peptide NYMAP1385 was injected. The cell loss and necrosis correlated with the areas of injection and did not appear to spread far beyond the site of injection. At 72 hours, cells appeared pale, shrunken, and necrotic and there was infiltration with inflammatory cells.

Apart from the mild areas of inflammation, controls showed no evidence of necrosis or cell loss. Control injections had mild to minimal acute inflammation at the injection sites and focal microhemorrhages from the needles.

The reduction in prostate volume in peptide NYMAP1385 injected rats was estimated to be on average 57.8% compared to controls (there was no discernible difference between control prostate volumes in control PBS injections alone, control inactive peptides, and no injections).

EXAMPLE 2

The purpose of this example was to determine the effect of peptide NYMAP1385 on tissue at sites of injection.

Seven normal male 300 gram weight Sprague-Dawley rats were given general anesthesia and given peptide NYMAP1385 by intraprostatic infusion. The injections consisted of 300 μl of peptide NYMAP1385 1 mg/mL in PBS pH 7.4. Controls received injections of PBS alone or no injection or other inactive peptides. Rats were painlessly sacrificed after 7 days. Prostate glands were dissected, fixed in 10% buffered formalin for 24 hours, embedded in paraffin, sectioned, and stained with H & E. For each animal the entire prostate gland was embedded and sectioned. All stained sections were examined histologically. For each animal the entire prostate gland was embedded and sectioned. All stained sections were examined histologically and measured. For each prostate at least 2 histological sections were examined, and for each histological section two cross-sectional diameters (D) at 90° from each other were measured (total of ≥4 measurements per prostate). The mean diameter from these measurements for each prostate was used to estimate volume according to

$$V=4/3*\pi*(D/2)^3$$

Results: Rat prostate treated with peptide NYMAP1385 showed necrosis of tissue at the injection sites with loss of glandular epithelium, flattening and atrophy. At 7 days, cells appeared pale, shrunken, and necrotic, and there was infiltration with inflammatory cells. There was no discernible difference between control PBS injections alone, and controls with no injections or control injections with inactive peptides.

The reduction in prostate volume in peptide NYMAP1385 injected rats was estimated to be on average 44.8% compared to controls (there was no discernible difference between control PBS injections alone, and controls with no injections).

EXAMPLE 3

The purpose of this example was to determine the effect of peptide NYMAP13134 (SEQ ID NO. 2) on tissue at sites of injection.

Nine rats were injected as in Example 1 above, except they were injected with peptide NYMAP 13134.

The animals were observed for 72 hours and painlessly sacrificed at 72 hours. Tissues were excised, fixed in 10% formalin, embedded in paraffin, and stained and examined by standard histopathological methods. For each animal the entire prostate gland was embedded and sectioned. All stained sections were examined histologically and measured. For each prostate at least 2 histological sections were examined, and for each histological section two cross-sectional diameters (D) at 90° from each other were measured (total of ≥4 measurements per prostate). The mean diameter from these measurements for each prostate was used to estimate volume according to

$$V=4/3*\pi*(D/2)^3$$

The controls were the same as Example 1.

Results: Injection of peptide NYMAP13134 produced cell death and necrosis of tissue at the injection sites. Similar to Example 1 above, the cell death was present in prostate and liver at the sites where peptide NYMAP13134 was injected. At 72 hours, cells appeared pale, shrunken, and necrotic and there was infiltration with inflammatory cells.

Apart from the mild areas of inflammation, controls showed minimal evidence of necrosis or cell loss. Control injections had mild to minimal acute inflammation at the injection sites and occasional focal microhemorrhages from the needles.

The reduction in prostate volume at 72 hours in peptide NYMAP13134 injected rats was estimated to be on average 72.5% compared to controls (there was no discernible difference between control PBS injections alone, and controls with no injections).

EXAMPLE 4

The purpose of this example was to determine the effect of peptide NYMAP13134 on tissue at sites of injection.

Eight normal male 300 gram Sprague-Dawley rats were injected in the prostate as in the above Example 2, except they were injected with peptide NYMAP13134. Rats were painlessly sacrificed after 7 days and their prostate glands were examined as in Example 2. For each animal the entire prostate gland was embedded and sectioned. All stained sections were examined histologically and measured. For each prostate at

least 2 histological sections were examined, and for each histological section two cross-sectional diameters (D) at 90° from each other were measured (total of ≥4 measurements per prostate). The mean diameter from these measurements for each prostate was used to estimate volume according to

$$V=4/3*\pi*(D/2)^3$$

Results: As in the above Example 2, injection of peptide NYMAP13134 produced significant cell loss and atrophy in the prostate at 7 days. At 7 days, cells appeared pale, shrunken, and necrotic, and there was infiltration with inflammatory cells, and there was extensive loss of glandular epithelium, flattening, and atrophy.

Controls showed minimal or absent changes, consisting of occasional mild focal inflammation from the needles.

The reduction in prostate volume in peptide NYMAP13134 injected rats at 7 days was estimated to be on average 75.2% compared to controls (there was no discernible difference between control PBS injections alone, and controls with no injections).

Histological changes were assessed on the following scale:

−Absent

+Present, Minimal

++Present, Moderate

+++Present, Moderate and Diffuse

++++Present, Diffuse and Extensive

Results: Table 4 below sets out the histological changes of cell death observed.

NYMAP Peptide	Histological Changes of Cell Death at 72 Hours	Histological Changes of Cell Death at 7 Days
1385	++++	++
13134	+++	++++
Controls	−/+	−

SEQUENCE LISTING

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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic peptide

<400> SEQUENCE: 1

Ile Asp Leu Leu Gln Gly Arg Thr Arg Asn Arg Cys
1 5 10

<210> SEQ ID NO 2
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<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic peptide

<400> SEQUENCE: 2

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<210> SEQ ID NO 3
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His His His His His His
1 5

What is claimed is:

1. A synthetic peptide consisting of the peptide selected from (i) Ile-Asp-Leu-Leu-Gln-Gly-Arg-Thr-Arg-Asn-Arg-Cys (SEQ ID NO. 1); or (ii) Phe-His-Asp-Leu-Lys-Lys-His-Cys-Ile-Lys (SEQ ID NO. 2) or salts thereof.

2. The synthetic peptide of claim **1**, wherein the peptide is Ile-Asp-Leu-Leu-Gln-Gly-Arg-Thr-Arg-Asn-Arg-Cys (SEQ ID NO. 1).

3. The synthetic peptide of claim **1**, wherein the peptide is Phe-His-Asp-Leu-Lys-Lys-His-Cys-Ile-Lys (SEQ ID NO. 2).

4. A composition comprising the peptide of claim **1**, and a carrier therefor.

5. A synthetic peptide of SEQ ID NO: 1 or SEQ ID NO: 2 with up to 25 additional amino acids flanking either the amino

or carboxy end of the peptide, wherein the peptide is cytotoxic.

6. The peptide of claim **5**, wherein the peptide is Ile-Asp-Leu-Leu-Gln-Gly-Arg-Thr-Arg-Asn-Arg-Cys (SEQ ID NO. 1).

7. The peptide of claim **5**, wherein the peptide is Phe-His-Asp-Leu-Lys-Lys-His-Cys-Ile-Lys (SEQ ID NO. 2).

8. A composition comprising the peptide of claim **5**, and a carrier therefor.

9. A method of treating a benign or malignant tumor in a mammal comprising locally administering to the mammal in need thereof a therapeutically effective amount of the peptide consisting of the sequence selected from: (i) Ile-Asp-Leu-Leu-Gln-Gly-Arg-Thr-Arg-Asn-Arg-Cys (SEQ ID NO. 1); or (ii) Phe-His-Asp-Leu-Lys-Lys-His-Cys-Ile-Lys (SEQ ID NO. 2).

10. The method of claim **9**, wherein the method is carried out on the mammal before, during, or after treatment of the mammal with a treatment selected from the group consisting of surgical excision, transplantation, grafting, chemotherapy, immunotherapy, vaccination, thermal or electrical ablation, cryotherapy, laser therapy, phototherapy, gene therapy, and radiation.

11. The method of claim **9**, wherein the tumor is a benign or malignant tumor of a tissue selected from the group consisting of lung, breast, stomach, pancreas, prostate, bladder, bone, ovary, skin, kidney, sinus, colon, intestine, stomach, rectum, esophagus; heart, spleen, salivary gland, brain and its coverings, spinal cord and its coverings, muscle, connective tissue, adrenal, parathyroid, thyroid, uterus, testis, pituitary, reproductive organs, liver, gall bladder, eye, ear, nose, throat, tonsils, mouth, and lymph nodes and lymphoid system.

12. The method of claim **9**, where the tumor is a hyperplasia of a tissue selected from the group consisting of lung, breast, stomach, pancreas, prostate, bladder, bone, ovary, skin, kidney, sinus, colon, intestine, stomach, rectum, esophagus, heart, spleen, salivary gland, brain and its coverings, spinal cord and its coverings, muscle, connective tissue, adrenal, parathyroid, thyroid, uterus, testis, pituitary, reproductive organs, liver, gall bladder, eye, ear, nose, throat, tonsils, mouth, and lymph nodes and lymphoid system.

13. The method of claim **9**, wherein said tumor is prostatic hyperplasia.

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